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Effects of the Herbicide San 9789 on Photomorphogenic Responses[1]

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ABSTRACT

The herbicide, 4-chloro-5-(methylamino)-2-(α,α,α-trifluoro-*m*-tolyl)-3(2H)-pyridazinone (San 9789), an inhibitor that prevents both carotenoid and chlorophyll accumulation and normal chloroplast development in white light, does not affect the physiological effectiveness of phytochrome in dark- and light-grown plants. Red/far red reversibility of growth inhibition, stimulation of anthocyanin synthesis, and stimulation of phenylalanine ammonia-lyase synthesis are not significantly different in plants grown with and without San 9789. Despite the complete absence of photosynthesis, flowering could be induced in the long day plant *Hordeum vulgare* L. when sucrose was provided to the leaves. Since the nonphotochemical reactions of phytochrome also are not affected by the herbicide, San 9789 may be used as a tool to study the phytochrome system spectrophotometrically in plants grown for relatively long periods under high intensity white light.

In many plant species sublethal doses of 4-chloro-5-(methylamino)-2-(α,α,α-trifluoro-*m*-tolyl)-3(2H)-pyridazinone (Norflurazon; hereafter referred to as San 9789, manufacturer's code no.) inhibit carotenoid synthesis (2). When exposed to white light these plants do not accumulate Chl pigments, but appear to grow and develop as well as untreated plants for several days.

Our interest in this herbicide evolved from the possibility of using it as a tool to study phytochrome spectrophotometrically in light-grown plants. Normally Chl fluorescence makes spectrophotometric measurements in light-grown plants impossible (18). Furthermore, R^3/FR-reversible-fluorescence yield changes, unrelated to phytochrome, produce apparent *A* changes which could be confused with phytochrome (6). Oat seedlings grown in the presence of San 9789 for 6 days have considerably less Chl than etiolated seedlings exposed to 1 min of light (12). Phytochrome concentration measured *in vivo* and *in vitro* in these herbicide-treated plants were found to be about 2% of the level in etiolated tissue. Thus, such plants can be used to circumvent the problems caused by Chl if the herbicide does not interfere with the phytochrome system itself.

It is the purpose of this publication to demonstrate that San 9789 effectively inhibits Chl accumulation, but does not affect the phytochrome system in dark- and light-grown plants. It will be shown that regulation of a number of growth responses, anthocyanin, and PAL-synthesis in dark- and light-grown plants and the photoperiodic control of flowering are under phytochrome control in herbicide-treated plants, as well as in the water controls.

MATERIALS AND METHODS

Plant Material. Barley (*Hordeum vulgare* L. cv. Wintex), oat (*Avena sativa* L. cv. Garry), rye (*Secale cereale* L. cv. Cougar), and white mustard (*Sinapis alba* L.) were used.

Wintex barley was grown at 21 C on Difco agar (0.5%) with and without 0.1 mM San 6706 for the growth rate experiments. For the flowering experiments, plants were grown at 20 C in Vermiculite subirrigated with Hoagland solution containing 0.1 mM San 6706. Sucrose (6%) was applied to the freshly cut ends of leaves throughout the course of the experiment. Oat and rye seedlings were grown at 25 C on one layer of Kimpack. Mustard seedlings were grown at 25 C using the standard techniques described elsewhere (14).

Herbicide. Unless otherwise indicated, all experiments were performed with San 9789. San 9789 is considered to be the metabolically active form of San 6706 (J. St. John, personal communication), but no difference was found in this study in the biological activity of San 9789 and San 6706 (the dimethylamino derivative of San 9789). San 9789 is sold commercially under the trade name Norflurazon or Zorial and was obtained from Sandoz-Wander, Inc., Homestead, Fla.

Light Sources. Monochromatic light was obtained from modified Withrow monochromators (9, 22) equipped with Baird-Atomic interference filters, 10 cm of water (+ 3.5% ferrous ammonium sulfate for FR), and Schott KG-1 heat absorption filters. Filter characteristics were: R, λ_{max}: 660 nm, halfwidth 8 nm; FR, λ_{max}: 730 nm, halfwidth 8 nm. White light was obtained from daylight fluorescent lamps (Sylvania 48T12/D/VHO). These lamps were supplemented with FR light by mixing them in a 1:1 ratio with single FR emitting phosphor fluorescent lamps (Westinghouse F48T12/IR/VHO) for the induction of flowering in barley. All of the physiological experiments were performed at the Smithsonian Radiation Biology Laboratory, except the PAL experiment and spectrophotometric measurements which were performed at the Institut für Biologie II in Freiburg, Germany. In the PAL experiment continuous irradiations with FR were obtained from a "standard" FR source (16) (emission maximum at 740 nm, bandwidth 123 nm, with an intensity or fluence rate of 3.5 w m^{-2}), and irradiations with white light were carried out in a phytochamber with xenon arc lamps (7,000 lux). FR monochromatic light was obtained from a xenon high pressure arc source (8) with an AL (band) interference filter (λ_{max} 756 nm, bandwidth 20 nm) from Schott (Mainz, Germany).

Physiological Measurements. In barley, the growth rate was determined with time lapse photography using a green safelight. Four 10-s exposures, 90 min apart, were given using Kodak

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[3] Abbreviations: R: red; FR: far red; P_{tot}: total phytochrome; PAL: phenylalanine ammonia-lyase; San 9789: 4-chloro-5-(methylamino)-2-(α,α,α-trifluoro-*m*-tolyl)-3(2H)-pyridazinone; San 6706: the dimethylamino derivative of San 9789.

Kodalith Ortho Film, type 3. Length measurements were made directly on enlarged images of the negatives.

The induction of flowering in barley under continuous fluorescent light supplemented with FR was compared to that under 8-h photoperiods in fluorescent light alone. Apices were dissected using a dissecting microscope and scored for floral stage (3) and then measured with a calibrated eyepiece reticle. Stage 1 refers to an indeterminate vegetative apex and stage 2 to a fully determinate reproductive apex that produces no further leaf primordia. Stages 3 to 10 are distinct morphological steps in the development of the mature influorescence.

Anthocyanin was extracted according to Lange *et al.* (13). Twenty 4-day-old rye seedlings were added to 20 ml extraction solution (propanol-HCl-H_2O, 19:1:80, vol. %) in glass vials, which were then immersed in boiling water for 7 min. For full extraction the seedlings remained in the extraction medium in the dark at 25 C for about 24 h. Extracts were centrifuged for 20 min at 20,000g and A was measured at 518 and 650 nm using a Beckman model 26 spectrophotometer. The A values at 518 were corrected for scattering (17):

$$\text{Corrected } A_{518} = A_{518} - 2.48\, A_{650}$$

All experiments contained four to six independent replicates. The standard errors are indicated.

PAL extracts were prepared from 20 mustard cotyledons, using a modification (21) of a method originally proposed by Zucker (23).

Spectrophotometric Measurements. *In vivo* measurements of phytochrome were performed with a custom built dual wavelength spectrophotometer described elsewhere (19). The measuring light was obtained through Schott DIL (line double) interference filters at 728 and 805 nm. Fifteen shoot tips were packed into a 6-mm i.d. metal cuvette. Each point in Figure 1 represents six to eight experiments. Phytochrome concentrations are given in relative units, normalized to the value at time zero = 100%.

RESULTS

Previously published results (12) clearly demonstrate the absence of carotenoid and Chl absorptions in light-grown barley plants treated with the herbicide San 9789. The absence of Chl is due to photooxidation since the Pchl synthesis and the transformation to Chl is not reduced in San 9789-treated seedlings. Phytochrome has been detected spectrophotometrically *in vivo* and *in vitro* in these seedlings at a level that is about 2% of that in etiolated seedlings.

A kinetic analysis shows that the phytochrome system in etiolated oat seedlings is not affected by the herbicide. The destruction rate of Pfr and the rate of new synthesis of Pr, after 3 h of white light, are not significantly different from the water control (Fig. 1). After destruction, the phytochrome concentration increases linearly at a slow rate during the following 2 days. The rate constant (K_s) for the resynthesis is $2.2 \times 10^{-4}\ \Delta\Delta\ A_{800}^{730} h^{-1}$.

In order to study the physiological effectiveness of the phytochrome system in herbicide-treated plants, we tested the R/FR reversibility of known photomorphogenic responses.

In etiolated barley plants grown with and without San 6706, inhibition of the growth rate by a brief R light pulse can be reversed by a subsequent FR light pulse (Table I). While the R/FR reversibility is almost 100% in both cases, the growth inhibition by R light is not as pronounced in herbicide-treated barley plants as in the controls.

The inhibition of mesocotyl elongation by Pfr, the physiologically active form of phytochrome, in dark-grown oats is not affected at all by San 9789 (Table II). The light treatment was given 3 days after sowing and repeated 24 h later, and mesocotyl length was measured in 5-day-old seedlings.

Anthocyanin synthesis has been reported to be stimulated by phytochrome (13, 17). As Table III demonstrates, incremental anthocyanin synthesis is under full phytochrome control in rye seedlings, grown with and without San 9789, in the dark. The light treatment was given 3 days after sowing and repeated 6 h later. Four-day-old seedlings were harvested and anthocyanin was extracted. The light-mediated anthocyanin synthesis was stimulated to a greater extent in the presence of the herbicide than in the water control, while the dark levels are the same in both. The large amount of anthocyanin present in herbicide-treated rye and mustard seedlings in continuous white light (unpublished data) clearly excludes any possibility of an involvement of photosyn-

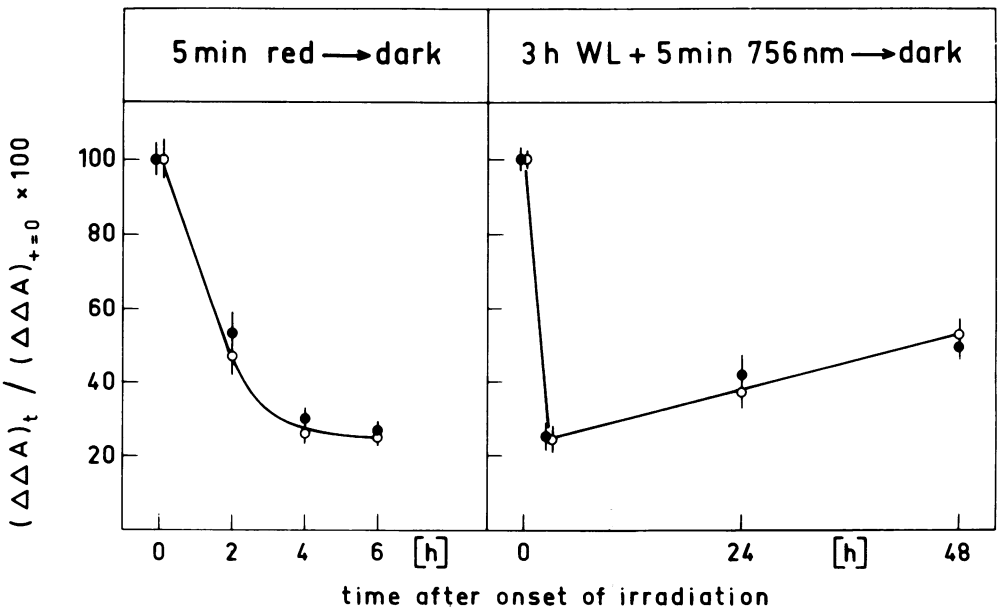

FIG. 1. Phytochrome changes, measured *in vivo*, in etiolated oat seedlings grown with (●) and without (○) 0.2 mM San 9789. Left: 5-day-old seedlings received 5 min of R (0.675 w m^{-2}) and were then returned to darkness (25 C). 100% = 0.035 ± 0.001 (San 9789); 0.032 ± 0.002 (water control). Right: 4-day-old seedlings were irradiated for 3 h with white light (xenon arc), before further decay was stopped with 5 min 756 nm light. 100% = 0.037 ± 0.002 (San 9789); 0.030 ± 0.002 (water control).

Table I. Effect of light pulse treatments on the growth rate of 3-1/2 day old wintex barley, grown with and without 10^{-4}M San 6706 (21 C).

Pretreatment	Control [mm/h]	+San 6706 [mm/h]
No pretreatment	1.19 ± 0.07	1.22 ± 0.07
90 s red[(a)]	0.89 ± 0.03	1.03 ± 0.08
90 s red + 90 s far red	1.20 ± 0.06	1.19 ± 0.05
90 s far red[(b)]	1.24 ± 0.05	1.10 ± 0.05
90 s green [(c)]	1.28 ± 0.04	1.21 ± 0.02

(a) 1.6 Wm^{-2}

(b) 4.7 Wm^{-2}

(c) 0.1 Wm^{-2} (time lapse photographic exposures totaled 90 s)

Table II. Control of mesocotyl elongation in dark-grown oat seedlings (25 C). Garry oats were grown with and without 10^{-4}M San 9789 for 5 days in darkness. Light pulses were given 72 h after sowing and repeated 24 h later.

Pretreatment	+San 9789 mm	Control mm
No pretreatment	69 ± 2	63 ± 2
30 s red[(a)]	28 ± 1	30 ± 1
30 s red + 150 s far red[(b)]	46 ± 2	47 ± 1
150 s far red	44 ± 2	49 ± 2

(a) 8.0 Wm^{-2}

(b) 6.0 Wm^{-2}

Table III. Control of anthocyanin synthesis in dark-grown rye (25 C). Rye was grown with and without 10^{-4}M San 9789 for 4 d in darkness. Light pulses were given 72 h after sowing and repeated 6 h later.

Pretreatment	Absorbance at 518 nm(x 10^{-3}) + San 9789	Control
No pretreatment	40 ± 5	37 ± 2
30 s red[(a)]	130 ± 11	89 ± 10
30 s red + 150 s far red[(b)]	55 ± 4	45 ± 3
150 s far red	56 ± 3	40 ± 5

(a) 8.0 Wm^{-2}

(b) 6.0 Wm^{-2}

thesis in the regulation of anthocyanin synthesis.

Phytochrome control (*i.e.*, R/FR reversibility) of the growth rate, is not only unaffected by the herbicide in dark-grown seedlings but also in light-grown plants (Table IV). Oat seedlings were grown for 8 days in 8/16 h light/dark cycles. The light quality, given at the end of the main light period determines the extent of mesocotyl elongation. According to the operational criteria for the involvement of phytochrome in a physiological response (15) this light acts through phytochrome without any significant difference between plants grown with and without San 9789.

The synthesis of the enzyme PAL is under the control of the phytochrome system in mustard seedlings (7). Since maximum activity of PAL under continuous FR light occurs 60 h after sowing, we compared the effect of 60-h darkness, continous FR, or white light on the PAL level in San 9789-treated mustard seedlings with the PAL level in the water controls. Table V indicates that the PAL levels do not differ significantly in plants grown with and without the herbicide. After 60 h of FR light there is almost twice as much PAL present as after 60 h of continuous white light. This is in agreement with the finding that the maximal HIR (high irradiance response) occurs under FR light.

Since herbicide-treated plants can only grow until they have exhausted their storage material, it is necessary to provide an exogenous carbohydrate source for longer term experiments. By applying sucrose through the cut ends of leaves it was possible to maintain barley for almost a month. In the absence of the herbicide the long day plant, wintex barley, flowers most rapidly under continuous light supplemented with FR while the 8-h short day controls without added FR do not flower during the course of the experiment. After growth for 22 days, some of the herbicide-treated plants reached floral stages as high as 4 with apex lengths of 14 mm in continuous light compared with the controls that never exceeded stage 1 with apex lengths no greater than 2 mm. This response is difficult to quantify since the technical difficulties involved with sucrose feeding through the leaves result in the loss of more than 80% of the samples during the course of the experiment. Those that survive are capable of flowering under long day inductive conditions but not under short day control conditions. The response is also considerably reduced when compared to those plants in continuous light that were not treated with the herbicide which after 22 days have reached stage 10 with apex lengths of about 40 mm. The controls with and without the herbicide were not markedly different. Growth of the herbicide-treated plants is also severely restricted, suggesting that the reduced flowering is due to the inability of the exogenous sucrose to adequately compensate for the loss of photosynthesis.

DISCUSSION

In a previous paper we suggested a method for measuring phytochrome in plants grown in white light (12). In that method we utilized the herbicide San 9789 which effectively inhibits Chl accumulation in white light. Results presented in this paper indicate that the phytochrome system is not affected by San 9789,

Table IV. Control of mesocotyl elongation in light-grown oats (25°). Garry oats were grown with and without 10^{-4} M San 9789 for 2 days in darkness and were then transferred to 8/16 h light/dark cycles. Additional light pulses were given at the end of main light period. Mesocotyl length was measured on 8 day old oats.

Light-treatment (per 24 h period)	+ San 9789 [mm]	Control [mm]
8 h WL[(a)]	4.9 ± 0.2	4.7 + 0.3
8 h WL + 90 s FR[(b)]	6.6 ± 0.3	6.9 ± 0.2
8 h WL + 90 s FR + 90 s R[(c)]	4.6 ± 0.2	4.9 ± 0.2

[(a)] day-light fluorescent 5,000 lux

[(b)] 2.5 Wm^{-2}

[(c)] 1.5 Wm^{-2}

Table V. Control of phenylalanine ammonia-lyase (PAL) activity in mustard cotyledons (25 C). PAL activity was determined in the cotyledons of 60 h old mustard seedlings grown with and without 5 x 10^{-6} M San 9789.

Treatment	p-mol transcinnamic acid / min · pair of cotyledons	
	+ San 9789	Control
60 h WL	31 ± 5	26 ± 2
60 h FR	56 ± 2	64 ± 5
60 h D	5 ± 1	3 ± 1

even if Chl is almost completely photooxidized. When studied spectrophotometrically, the level of phytochrome in dark-grown oat seedlings is not significantly altered, and the nonphotochemical reactions (*i.e.* Pfr destruction and Pr synthesis) are not affected by San 9789. While these reactions have been studied only in oat seedlings, results to be presented elsewhere suggest that the phytochrome system, studied spectrophotometrically, is not affected by San 9789 in other plants as well.

The phytochrome-mediated regulation of mesocotyl elongation is not different in etiolated oat seedlings grown with and without San 9789. However, in Wintex barley, the inhibition of the growth rate by R is not as pronounced in herbicide-treated plants, while its R/FR reversibility is normal. Similar results were found with albino barley seedlings (5). Action spectra for the inhibition of stem growth did not differ significantly in dark-grown seedlings of albino and nonalbino barley. Since the San 9789 (San 6706)-treated plants and the carotenoid-free albino mutants (1) behave similarly with regard to this phytochrome response, it is suggested that the herbicide acts primarily at the level of the chloroplast and does not cause major disruptions of other aspects of cellular metabolism. Such chloroplast specificity in the mode of action of this herbicide has been demonstrated in a number of other systems (1, 2, 10, 11, 20).

Anthocyanin synthesis is stimulated to a greater extent by R light in herbicide-treated rye seedlings compared to the water controls, but, the R/FR reversibility is 100% in both cases. The herbicide does not seem to affect the phytochrome control of plant development at the molecular level, since the synthesis of the enzyme PAL is not significantly different from the water control under three different light conditions.

The situation does not seem to be different in light-grown plants. Table IV shows that the light quality at the end of the main light period is important for mesocotyl elongation in the subsequent dark period. A high Pfr/P_{tot} ratio at the beginning of the night inhibits mesocotyl elongation more than a low Pfr/P_{tot} ratio. These data suggest that mesocotyl elongation does not depend on functioning chloroplasts and photosynthesis (as long as the plant has enough storage material). The control of mesocotyl elongation during the daily light period is due solely to a blue receptor(s) and/or phytochrome.

Photosynthesis is also not essential for the photoperiodic induction of flowering in Wintex barley when sucrose is provided exogenously. Although it is technically very difficult to maintain plants for time periods long enough to evaluate flowering responses and the majority of plants are lost, it is nevertheless clear that herbicide-treated plants are capable of being induced to flower by long days. The role of photosynthesis in this long day plant is solely to provide sufficient photosynthate for the maintenance of plant growth and the induction of flowering is strictly controlled by a nonphotosynthetic (and noncarotenoid) photoreceptor, presumably phytochrome (4).

In all physiological responses studied so far, we find that the herbicide San 9789 does not affect the R/FR reversibility of the responses. Therefore, San 9789 does not appear to interfere with the phytochrome system in any way. Neither the phytochrome system not the photomorphogenic responses are different in dark-grown treated and untreated seedlings. Since photomorphogenic responses are also not affected by San 9789 in light-grown plants, we conclude that the nonphotochemical reactions of the phytochrome system in light-grown tissue are also not affected by the herbicide San 9789. Thus, we suggest that the phytochrome system in these herbicide-treated plants is essentially the same as the phytochrome system in green plants grown without the herbicide.

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LITERATURE CITED

1. Bartels PG, A Hyde 1970 Chloroplast development in 4-chloro-5-(dimethylamino)-2-(α,α,α-trifluoro-*m*-tolyl)-3(2H)-pyridazinone (Sandoz 6706)-treated wheat seedlings. Plant Physiol 45: 807–810
2. Bartels PG, C McCullough 1972 A new inhibitor of carotenoid synthesis in higher plants: 4-chloro-5-(dimethylamino)-2-(α,α,α-trifluoro-*m*-tolyl)-3(2H)-pyridazinone (San 6706). Biochem Biophys Res Commun 48: 16–22
3. Bonnett OT 1935 The development of the barley spike. J Agric Res 51: 451–457
4. Borthwick HA, SB Hendricks, MW Parker 1948 Action spectrum for photoperiodic control of floral initiation of a long day plant, Wintex barley (*Hordeum vulgare*). Bot Gaz 110: 103–118
5. Borthwick HA, SB Hendricks, MW Parker 1951 Action spectrum for inhibition of stem growth in dark-grown seedlings of albino and non-albino barley (*Hordeum vulgare*). Bot Gaz 113: 95–105
6. Butler WL 1962 Effects of red and far-red light on the fluorescence yield of chlorophyll *in vivo*. Biochim Biophys Acta 64: 309–317
7. Dittes L, I Rissland, H Mohr 1971 On the regulation of enzyme levels (phenylalanine ammonia-lyase) in different organs of a plant (*Sinapis alba* L.). Z Naturforschung 26b: 1175–1180
8. Hartmann KM 1967 Ein Wirkungsspektrum der Photomorphogenese unter Hochenergiebedingungen und seine Interpretation auf der Basis des Phytochroms (Hypokotyl-Wachstumshemmung bei *Lactuca sativa* L.). Z Naturforschung 22b: 1172–1176
9. Hayes R, WH Klein 1974 Spectral quality influences of light during development of *Arabidopsis thaliana* plants in regulating seed germination. Plant Cell Physiol 15: 643–653
10. Hilton JL, JB St John, MN Christiansen, KH Norris 1971 Interactions of lipoidal materials and a pyridazinone inhibitor of chloroplast development. Plant Physiol 48: 171–177
11. Hilton JL, AL Scharen, JB St John, DE Moreland, KH Norris 1969 Modes of action of pyridazinone herbicides. Weed Sci 17: 541–547
12. Jabben M, GF Deitzer 1978 A method for measuring phytochrome in plants grown in white light. Photochem Photobiol 27: 799–802
13. Lange H, W. Shropshire Jr, H Mohr 1971 An analysis of phytochrome-mediated anthocyanin synthesis. Plant Physiol 47: 649–655
14. Mohr H 1966 Untersuchungen zur Phytochromeinduzierten Photomorphogenese des Senfkeimlings (*Sinapis alba* L.). Z Pflanzenphysiol 54: 63–83
15. Mohr H 1972 Lectures on Photomorphogenesis. Springer, Berlin
16. Mohr H, E Pinnig 1962 Der Einfluss des Lichtes auf die Bildung von Blattprimordien am Vegetationskegel der Keimlinge von *Sinapis alba* L. Planta 58: 569–579
17. Ping-Kaung K, AL Mancinelli 1972 Photocontrol of anthocyanin synthesis. Plant Physiol 49: 212–217
18. Pratt LH 1978 Molecular properties of phytochrome. Photochem Photobiol 27: 81–105
19. Pratt LH, D Marmé 1976 Red light-enhanced phytochrome pelletability. Plant Physiol 58: 686–692
20. St John JB 1976 Manipulation of galactolipid fatty acid composition with substituted pyridazinones. Plant Physiol 57: 38–40
21. Tong Wu-Fu 1975 Untersuchungen zum Mechanismus der phytochrom-induzierten Akkumulation von Phenylalaninammoniumlyase in den Kotyledonen des Senfkeimlings (*Sinapis alba* L.). PhD thesis. University, Freiburg
22. Withrow RB 1957 An interference-filter monochromator system for the irradiation of biological material. Plant Physiol 32: 355–360
23. Zucker M 1965 Induction of phenylalanine deaminase by light and its relation to chlorogenic acid synthesis in potato tuber tissue. Plant Physiol 40: 779–784